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sof GENE AS A SPECIFIC GENETIC MARKER FOR DETECTION OF *Streptococcus pyogenes* CAUSING PHARYNGITIS AND RHEUMATIC HEART DISEASE

A. KUMAR[1], A. BHATNAGAR[1], S. GUPTA[2], S. KHARE[2] AND SUMAN[3]

1 Institute of Genomics and Integrative Biology (CSIR), Mall Road, Delhi-110007, India
2 National Centre for Disease Control, Sham Nath Marg, Delhi-110054, India
3 Institute of Advance Research and Studies, Amity University, Noida-201303, India

Abstract

Streptococcus pyogenes is a human pathogen causing invasive and non-invasive diseases, as well as severe sequels, such as rheumatic fever. Rheumatic heart disease is a sequel of rheumatic fever results from an untreated strep throat causing damage of the heart valves. The usual detection methods of strep throat are culture, virulent test, antibiotic sensitivity, CRP, ESR and PCR. These methods are expensive, time consuming and have some limitations. All reported PCR methods are based on either 16S rRNA or specific gene based along with other methods to confirm the disease in more than 1h. Here, we have developed a PCR based diagnosis of *streptococcus pyogenes* using specific primers of virulent *sof* gene (serum opacity factor) of *S. pyogenes.* Our method is an improvement of the existing methods and the overall analysis completes in 1 h which is the least time reported so far for the confirmation of the disease. Amplicon of 228 bp of *sof* gene does not show homology with other organisms and can be used as genetic marker for *S. pyogenes.*

Key words: Genetic marker, Pharyngitis, Rheumatic heart disease, *S. pyogenes*, *sof* gene.

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Corresponding author
Ashok Kumar
Institute of Genomics and Integrative Biology (CSIR), Mall Road, Delhi-110007, India
Fax: 91-11-27667471
E-mail: ashokigib@rediffmail.com

Abbreviations: BLAST : Basic local alignment search tool; **EDTA** : Ethylenediaminetetraacetic acid; **mga** : multiple gene activator; **SOF** : serum opacity factor; **RH** : rheumatic fever; **RHD** : rheumatic: heart disease; **VCAM** : vascular cell adhesive molecule.

INTRODUCTION

Streptococcus pyogenes (Group A streptococcus) is a Gram-positive, nonmotile and non spore forming coccus. The group A streptococcus (GAS) causes a variety of diseases, ranging from mild and self-limiting infections of the pharynx and skin to more-severe and life-threatening infections. The major sequels of group A *streptococcal* infections are acute rheumatic fever (RF), rheumatic heart disease (RHD) and acute glomerulonephritis (4). The incidence of rheumatic fever has decreased somewhat in developed countries but still the major problem in developing countries specially in school children. The prevalence rate of rheumatic heart disease in India is around 6-11 cases whereas in US 0.6, Japan 0.7, Asia 0.4-21, 0.3-15 and south Africa 1-17 cases per 1000 school children population (19). Approximately 10,000-15,000 cases of invasive GAS disease occur annually in the United States, associated

with a 10-13% mortality rate (2). *Streptococcus pyogenes* produces a wide array of virulence factors that helps the pathogenesis of this bacterium in the host viz M protein, R and T protein, F-protein, streptolysin-A, streptolysin-O, peptidoglycan, lipoteichoic-acid, hyaluronidase, protease, **s**treptodornases A-D, superantigens (5,12,16). Rapid and accurate diagnosis is essential for both optimal management of patients and for timely antibiotic mediated prophylaxis. The commonly used diagnostic tests for *S. pyogenes* are culture, immunological test, biochemical test and PCR The advent of PCR technology has positive impact on biomedical research by providing the most sensitive and rapid method to detect microbial pathogens in clinical samples.

It was evident that GAS are related with heart disease due to autoantibody responses (cross reactivity) but since there are many antibodies in the sera, monoclonal antibodies were used to understand the pathogenesis of RHD, that showed the reaction of MAbs with myocardium in heart tissues (7-9).Rheumatic fever is a systemic disease affecting the peri-arteriolar connective tissue and can occur after an untreated Group A ß- hemolytic streptococcal pharyngeal infection. It may be due to antibody cross-reactivity Type II hypersensitivity reaction and is termed *molecular mimicry* (1, 14). RF leads to Rheumatic heart disease. Initially, the organism adhere and invade host epithelial cells, then B and T cells are activated by specific streptococcal antigens and superantigens leading to strong responses against streptococcal and host antigens. The development of pathogenic clones of B and T lymphocytes are important in development of the disease. The antibodies against the group A carbohydrate, which is cross-reactive with the valve surface, bind to the valve surface endothelium (endocardium) and lead to damage of the valve. M protein-reactive T cells enter the valve through the surface endothelium by binding to cell adhesion molecules such as VCAM-1 and extravasate into the valve. The formation of scar tissue in the valve followed by neovascularization allows for the disease to continue in the valve (10, 11). Identification of serum opacity factor (*sof)* gene which serves as a marker for serotyping and *S. pyogenes.* SOF Binds to Fibulin-1 and fibrinogen present in serum, this search was undertaken because many of the surface proteins of group A streptococci have multiple binding domains for serum proteins, and the binding of these proteins have been linked to increased survival of group A streptococci in blood and to adhesion to host cells (3,6,15). Serum opacity factor is a bifunctional cell surface protein expressed by 40-50% of group A streptococcal strains comprised of a C-terminal domain that binds fibronectin and an N-terminal domain that mediates opacification of mammalian sera (17, 21). The molecule SOF exhibits N terminal sequence variation and is under the positive transcriptional regulation of *mga* (multiple gene activator) and elicit type-specific immune responses (13).

MATERIALS AND METHODS

Sample collection and chemicals

The bacterial culture was from IMTECH Chandigarh and the patients throat swab samples were collected from Safdarjang Hospital, Delhi. dNTP, Taq polymerase, PCR buffer, $MgCl_2$ and RNase were from Bangalore GeNei, India. Tris and EDTA were purchased from Sigma-Aldrich, USA. DNA purification kit (GFX column) was purchased from Amersham Biosciences, UK Ltd. Primers were synthesized from TCGA (The Centre for Genomic Application), India.

Sample preparation

The patient throat swab sample was directly dissolved in 1 ml of STE buffer (50 mM Tris, 50 mM EDTA, 20% sucrose, pH 8.0) and heated for 2 min at 95°C then centrifuged at 3,800xg for 2 min. The pellet was washed twice with milli Q water by centrifugation and finally dissolved in water and transferred into PCR tube. This was used as genomic DNA and quantified by Nanodrop spectrophotometer. The *S. pyogenes* (strain M140, IMTECH, India) was also cultured in Todd Hewitt broth and genomic DNA was isolated at NCDC using phenol chloroform method (18) to use as control.

Amplification of target gene

The genome of *Streptococcus pyogenes* was obtained from the NCBI and the sequence of *sof* gene was retrieved and checked for the homology with genes of other organisms. The specific *sof* gene based forward primer (5'-TAGCCCCGACAGTT TTAGGA-3') and reverse primer (5'-AGGCTGGAGTAGTGCCTGAA-3') were synthesized and PCR was carried out with the following steps: Initial heating at 95°C for 2 min, followed by 25 cycles of denaturation at 95°C for 7s, annealing at 55°C for 10s, extension at 72°C for 20 s and final extension for 3 min after the last cycle. The PCR was performed in 25µl of reaction mixture containing 1 x assay buffer, 1mM of dNTP (0.25mM of each dATP, dGTP, dCTP and dTTP), 0.2 µM each of forward and reverse primers, genomic DNA (approx.100ng), 0.75 units Taq polymerase and Milli Q water. Sharp band was observed corresponding 228 bp in DNA marker. The resulting PCR product was purified using GFX column and electrophoresis of PCR product was carried out in 2 % agarose gel. The PCR product was

sequenced by TCGA (The centre for genomic application) and homology of the sequence was confirmed by BLAST.

RESULTS AND DISCUSSION

The amplicon of 228 bp was viewed in 2 % agarose gel in UV light (Fig.1). The DNA sequence of amplicon with BLAST shows similarity with *sof* gene of *S. pyogenes* which confirmed that the right fragment within *sof* gene had been correctly amplified. *sof* is a unique virulence gene of *S. pyogenes* and therefore, PCR based detection of *S. pyogenes* using this gene has higher specificity. SOF had proven to play important role in fibulin binding, opacifying the serum and adhesion, of the pathogen to the epithelial cells of the host (6). Many methods like culture, CRP (c-reactive protein), EST (erythrocyte sedimentation test), rapid antigen test, antibiotic sensitivity are available but these are time consuming and have some limitations (22). Here, we have used PCR as a powerful tool in detection of *S. pyogenes* in 1 h (including electrophoresis) without isolating genomic DNA from pathogen. Primers used in this experiment are specific and amplify only the specific region under specific conditions. Since *sof* is virulence gene and does not have homology with other organisms, it can be used as genetic marker (228bp) for the detection of *S. pyogenes* causing pharyngitis and rheumatic heart disease. The diagnosis of control and suspected bacterial pharyngitis patients (20 samples) were carried out using different available methods (Table 1) as well as PCR using *sof* gene primers (Fig.2).

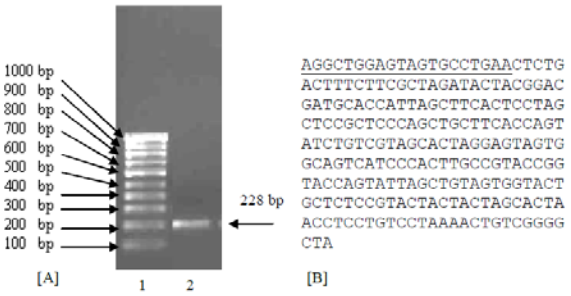

Figure 1. (A) Agarose gel electrophoresis (2.0%) of PCR product.Lane1: DNA Ladder 100bp; Lane2: purified PCR product (228 bp). (B) Gene sequence of PCR product (228bp) using forward primer.

Table 1. Diagnosis of suspected patients using different available methods and PCR using *sof* gene as specific genetic marker

Sample No.	*Present available methods*					*PCR with marker*	Normal/ Infected patients
	Microscopic (Gram +/-)	Immunology (Rapid Ag detection)	Culture on blood agar ß-hemolysis	Enzyme test (catalase)	Antibiotic succeptibility (Bacitracin)	*sof* gene (228 bp)	
1 control	+	+	+	-	+	+	C
2 control	+	+	+	-	+	+	C
3	-	-	-	-	-	-	N
4	-	-	-	-	-	-	N
5	+	+	+	+	-	+	**P**
6	+	+	+	-	+	+	**P**
7	-	-	-	-	-	-	N
8	-	-	-	-	-	-	N
9	-	-	-	-	-	-	N
10	-	-	-	-	-	-	N
11	+	+	+	-	+	+	**P**
12	-	-	-	+	-	-	N
13	-	-	-	-	-	-	N
14	-	-	-	-	-	-	N
15	-	-	-	-	-	-	N
16	-	-	-	-	-	-	N
17	-	-	-	-	-	-	N
18	-	-	-	-	-	-	N
19	-	-	-	-	-	-	N
20	-	-	-	-	-	-	N

Normal healthy individuals = N Control =C

S.pyogenes infected patients = **P**

Sample No.5, 6 and 11 were confirmed positive by PCR whereas it was showed negative by other methods due to their limitations. Sample No.5 and 12 showed catalase positive whereas others showed negative. Catalase test is not specific for *S. pyogenes* because *S. pyogenes* strains has divided into two classes with respect to the production of H_2O_2, i.e., producers and nonproducers (20). However, the metabolic basis and biological significance of bacterial H_2O_2 production are largely unexplored. Similarly, other tests have also some limitations and sometimes give false results. The results suggest that *sof* gene can be used as specific genetic marker.

Figure 2. Agarose gel electrophoresis (2.0%) of normal and infected patients with pyogenes. Lane 1 and 2 (control) and lane 5, 6 and 11 show single band corresponding to 228 bp of DNA marker (infected patients) whereas Lane 4, 7-10, 12-20 showed no bands (normal person).

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Other articles in this theme issue include references (23-38).

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